

An Analysis of the Role of Skin Langerhans Cells (LC) in the Cytoplasmic Processing of HIV-1 Peptides After "Peplotion" Transepidermal Transfer and HLA Class I Presentation to CD8⁺ CTLs—An Approach to Immunization of Humans

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Received February 28, 1994; accepted April 19, 1994

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Abstract. Skin Langerhans cells (LC) are antigen-presenting cells capable of expressing MHC class I and class II molecules on the plasma membrane. This molecular activity was reviewed to combine the knowledge of peptide presentation by MHC and HLA class I and class II molecules to prime CD8⁺ cytotoxic T cells (CTLs) and CD4⁺ T helper cells, respectively. The possible utilization of the skin dendritic cells for the development of antiviral CTLs and antibodies by synthetic peptides modeled according to the motifs of peptides that naturally interact with the peptide binding grooves of the various HLA haplotypes is discussed and evaluated. It may be possible that the introduction of synthetic viral peptides with motifs to fit the HLA class I haplotypes of a human population to the skin dendritic cells will prime selectively the cellular or the humoral immune responses. This approach may provide a new vaccination technique that applies synthetic virus peptides as vaccines for the immunization of humans. The neuropeptide CGRP interacts with LC and modulates antigen presentation.

Key words: immunization of human, synthetic viral peptides, skin Langerhans cells (LC), properties of LC, molecular aspects

Introduction

Immunization of humans against viral diseases has markedly advanced during the second half of the 20th century with the introduction of killed virus vaccines, attenuated virus vaccines, and genetically engineered virus vaccines. The establishment of an infection in humans by nonpathogenic attenuated viruses leads to an infectious process similar to natural infection with pathogenic viruses. This process causes the immune system of the immunized host to respond with the production of both antiviral antibodies and cytotoxic T cells (CTLs), as well as by induction of cytokines and immune memory cells, which provide long-term immunological protection. In

contrast, utilization of killed virus vaccines for immunization leads to the introduction of virus antigens, which stimulate the immune response (without establishing a virus infection) to synthesize antiviral antibodies. In the last 10 years attempts have been made to replace the killed virus vaccines with viral synthetic peptides that may induce synthesis of protective antibodies in the host capable of neutralizing infectious virus. However, synthetic virus vaccines that induce cytotoxic T cells are not yet available. The CTLs are capable of recognizing virus-infected cells in vivo by a human leukocyte antigen (HLA)-controlled mechanism, followed by their attachment to the infected cells, thus causing the death of the virus-infected cell prior to virus release.

In animal experiments, the best results with synthetic viral peptides as antigens were achieved by the injection of such peptides into the skin (subcutaneously or intradermally). Since epidermal Langerhans cells (LC) are present in the skin all over the body and are known to be efficient antigen-presenting cells, it may be possible to apply synthetic viral peptides directly to the skin in a lotion ("Peplotion"), which would allow the entry of the peptide into the layer of skin where the LC are present.

It was shown that LC interact with a hapten, an antigen, or a virus, migrate to the regional lymph nodes, and form clusters with T cells, presenting the antigens to the T cells. Introduction into human skin of synthetic peptides with motifs to bind to the recipient's HLA class I molecules will selectively prime cytotoxic T cells (CTLs). The immune defense mechanism of the immunized individual will be stimulated without the synthesis of antiviral antibodies. Such an approach is needed in virus infections that are enhanced by the antiviral antibodies, such as HIV-1, flaviviruses, and respiratory virus infections.

The aim of the present study is to explore the current knowledge on the role of LC and dendritic cells (DC) in immune processes and the molecular mechanisms that involve presentation of peptides to cytotoxic T cells and T helper cells by major histocompatibility complex (MHC) class I and class II molecules, respectively. It will be shown that skin LC are capable of interaction with synthetic peptides with MHC class I or class II motifs and that utilization of these cells for immunization with synthetic viral peptides with motifs to bind to the HLA class I molecules ensures the effective production of CTLs.

Skin LC Function as Antigen-Presenting Cells

Properties of LC

The LC in the skin are generated in the bone marrow from stem cells defined as dendritic/LC colony-forming units (1). It was found that macrophages and dendritic/LC share a common

bone marrow stem cell (1). The release of DC to the blood and their migration to the skin, first to the dermis and then to the epidermis by chemotaxis, was recently discussed by Knight et al. (2) and Bergstresser et al. (3). LC are positioned among the keratinocytes in a single layer above the stratum granulosum. The involvement of LC in the regulation of the proliferation of the skin keratinocytes has been suggested (4).

Response of LC to Haptens: Uptake of Haptens and Migration to the Afferent Lymph Nodes

Hapten painting of the mouse skin was reported to stimulate LC migration from the skin epidermis through the draining lymph nodes in the dermis to the regional lymph node (5). Painting of human skin with dinitrochlorobenzene led to an increase in CD1⁺ cells in the afferent lymph draining the site (2).

Painting of mouse footpad skin with the contact sensitizer fluorescein isothiocyanate (FITC) and preparation of the epidermal sheets for observation in the UV microscope revealed that FITC is present only in association with LC, while the keratinocytes were not stained by this hapten. Shortly after FITC treatment, fluorescein-labeled DC were found in the afferent lymph node cells (Becker, Sprecher, and David, unpublished). This observation is in agreement with Macatonia et al. (6) and Knight et al. (2), who studied the effect of a second exposure of mice to FITC 1 month after an initial exposure. It was found that a second exposure to FITC caused significantly lower numbers of DC carrying the antigen to the lymph node as well as a reduced T-cell proliferation.

The direct route of LC migration from the skin into the dermis was established in an organ culture system. Larsen et al. (7) reported that the majority of the migrating leukocytes were Ia⁺ (HLA class II⁺) LC and the remainder of the cells were comprised of Thy-1⁺, CD3⁺, CD4⁻, CD8⁻, γ/δ ⁺ receptor epidermal dendritic T cells that clustered with LC and a small population of macrophages. It was suggested that the maturation of LC commences in the epidermis and continues during LC migration.

*Interaction of LC with Biological Antigens:
Proteins, Viruses, and Leishmania*

Since LC are situated in their specific layer among keratinocytes, they may provide the vertebrate with a defense mechanism against foreign biological substances that enter the body through the skin. Such antigens may include proteins and infectious viruses entering through the damaged skin, as well as viruses and protozoa released into the skin with the saliva of biting insects. In studies on herpes simplex virus-1 (HSV-1) infection in the mouse skin (8) it was demonstrated that in the absence of LC at the site of virus injection, HSV-1 strains that were not pathogenic to the mouse caused a lethal infection. However, when LC in the skin at the site of virus injection were treated with the immunomodulator OK432 (a streptococcal cell wall preparation), even highly pathogenic HSV-1 strains did not cause a lethal infection. These results established a role for LC in defense against HSV-1 infection. A similar observation was reported for vaccinia virus infection in mice (8). The ability of viruses to interact with LC was recently reviewed (9,10).

It was also reported that saliva proteins released during a tick bite into the skin are present only on LC in the skin (11), indicating the ability of cell surface molecules on LC to interact with the foreign proteins. The ability of *Leishmania major* to infect LC was reported by Domp Martin et al. (12).

Epidermal LC Have CD1 Molecules on the Plasma Membrane and Internalize and Recycle the Surface CD1 Molecule by Receptor-Mediated Endocytosis

Dendritic cells express CD1 (MHC class I-like) glycoproteins on the plasma membrane. The CD1 antigen has a structure similar to HLA class I and is present on the cell membrane in association with a β_2 -microglobulin molecule. Hanau et al. (13,14) used immunogold staining of human epidermal Langerhans cells by anti-Tc antigen to identify the LC membrane-associated antigen, OKT6. These authors observed that the T6 molecules to which the antibody is attached were in-

ternalized via coated pits, coated vesicles, and endosomes. Intracytoplasmic Birbeck granules that contain the T6 antigen may represent intracellular transport organelles carrying the T6 antigen from the Golgi apparatus to an unknown destination. Hanau et al. (13,14) suggested that the T6-antigen on the plasma membrane of LC may be part of a receptor site. The T6 antigen may be a cell surface glycoprotein, CD1, associated with a β_2 -microglobulin resembling HLA class I antigen of the major histocompatibility complex (MHC) (15,16), which presents peptides to T cells with γ/δ receptors (17).

Dendritic Cells Express HLA Class I Molecules on the Plasma Membrane

All the dendritic cells express HLA class I molecules (17), which consist of heterodimers of a highly polymorphic α chain associated with a β_2 -microglobulin molecule (18). The expression of the less polymorphic nonclassical murine class I molecules Qa-2.3 and T1a by dendritic cells has not yet been evaluated (17).

Dendritic Cells Have MHC Class II Antigens on the Plasma Membrane

Knight et al. (19) reported that human blood dendritic cells are highly HLA class II positive. The anti-HLA-DR antibody labeled with 20-nm gold particles revealed that the MHC class II molecules can be seen on the plasma membrane, in cytoplasmic vacuoles near the plasma membrane, and in cytoplasmic vacuoles near the nuclei (2) (possibly the endoplasmic reticulum ER lumen), demonstrating the pathway of the HLA class II molecules from the ER to the plasma membrane.

Prue et al. (20) reported that antigen processing by LC correlates with the level of MHC class II and invariant chain (Ii) biosynthesis. Similarly, Kampgen et al. (21) reported that freshly isolated LCs synthesize large amounts of class II and invariant chain (Ii) polypeptides. The turnover of the MHC class II polypeptides is slow, while the turnover of the Ii polypeptide is rapid. This result indicates that the MHC α and β chains and the Ii polypeptides are efficiently

transported from the membrane of the ER to the endosome pathway. At that site, the vesicle that transports the MHC class II molecules fuses with the endosomes, which contain the proteolytic enzyme cathepsin. As the result of the fusion the cathepsin proteolytically cleaves the Ii chain. The in vitro cultured LC lose their capacity to process exogenous antigens but retain their ability to prime T cells. Culturing LC resulted in the disappearance of endosomes and acidic granules under the in vitro conditions that accompanied the loss of antigen processing (22).

Synthesis of I-A α Chain mRNA in LC

Tang et al. (23) reported that it was possible by the PCR technique to demonstrate that mouse LC expressed the I-A α gene. Expression of MHC class II antigen by cultured LC in vivo activated with haptens was reported by Aiba and Katz (24). Using anti-I-A antibodies a two- to three-fold increase in I-A and I-E expression by LC was shown, while CD45 antigen expression was not altered. Two subpopulations of LC were noted: LC with an unchanged Ia density, and 20–50% of LC with a markedly enhanced Ia density (10-fold increase in I-A and I-E). This change was caused only by haptens.

LC Plasma Membrane Glycoproteins

Membrane Ecto-ATPase on Epidermal LC

Epidermal LC possess on their cell membrane a formalin-resistant ATPase activity (25,26). This ATPase is an ectoenzyme with an active site on the extracellular side of the plasma membrane and requires Mg^{2+} or Ca^{2+} for activation. Girolomoni et al. (27) reported that human LC are resistant to the permeabilizing affects of ATP and suggested that the membranal ATPase provides protection to LC.

Cytokine Production by Epidermal LC

Matsue et al. (28) reported that LC from trypsinized mouse epidermal sheets contained PCR-detectable mRNA of the cytokines IL-1 β and TNF- α . FACS-purified Ia $^{+}$ EC expressed IL-1 β

and macrophage inflammatory protein-1 α (MIP-1 α). The production of IL-1 β and TNF- α by mouse skin LC was reported by Sprecher and Becker (29,30).

Human Epidermal LC Express Integrins of the β 1 Subfamily

Le Varlet et al. (31) and Staquet et al. (32) reported that normal human LC were integrin β 1 (CD29)-positive by indirect immunogold labeling on transmission electron microscopy. By labeling with different VLA- α chain (CD49) monoclonal antibodies, two subpopulations of LC were detected: About 40% had small amounts of VLA-1 and VLA-3, and 53% and 77% of LC expressed moderate amounts of VLA-2 and VLA-5, respectively. VLA-4 and VLA-6 were expressed by 67% and 90% of LC, respectively. VLA-6, the laminin receptor and VLA-5, the fibronectin receptor, are expressed by LC, possibly to help the LC during migration throughout the fibronectin network of the dermis before reaching the afferent lymphatics on the way to the regional lymph nodes. It was suggested by Staquet et al. (32) that the LC that attach to laminin and fibronectin in vitro mediated the passage of LC through the basement membrane and through the fibronectin network of the dermis by the α 5 β 1 and α 6 β 1 surface molecules.

Adhesion of Epidermal LC to Keratinocytes Is Mediated by E-Cadherin

Tang et al. (23) reported that mouse keratinocytes express E-cadherin, a member of the cadherin family that is represented in epithelial and neural tissues and plays an important role in the morphogenesis and maintenance of tissue integrity (33). It was demonstrated that LC contain E-cadherin mRNA and were able to bind to cells that express E-cadherin on their surface (L-cells transfected with E-cadherin cDNA).

Fc γ Receptors Expressed by Human Epidermal LC

Schmitt et al. (34,35) reported that in healthy individuals 50% of CD1a-positive epidermal LC

express the Fc γ RII/CDw32 receptor for IgG, which has a molecular weight (M_r) of 40 kD. In atopic dermatitis patients with elevated IgE in the serum, about 50% of epidermal CD1-a positive cells present in lesional skin are IgE bearing. It was found that IL-4 and/or interferon- γ induce the expression of IgE receptors FcERII/CD23 on a percentage of normal human epidermal LC. The gold-labeled monoclonal antibodies to FcRIII were internalized by epidermal LC by receptor-mediated endocytosis and accumulated in the cell lysosomes.

Dendritic Cells Prime Cytotoxic CD8⁺ T Cells by MHC Class I Restriction

"Extraordinary Capacity" of Allogeneic Epidermal LC to Prime Cytotoxic T Cells

McKinney and Streilein (36) examined the relative alloimmunogenicity of epidermal Langerhans cells, Thy-1⁺ dendritic epidermal cells, and keratinocytes prepared from mouse skin. Each cell type was injected intravenously (IV) or subcutaneously (SC) into allogeneic recipient mice. At 4–6 weeks spleens and lymph nodes of recipient mice were assayed for specific priming of cytotoxic T cells. LC were found to be powerful immunogens, since only LC (at a dose of 10 LC) primed allospecific T cells of mice that received the injected cells.

Dendritic Cells Present Sendai Virus Antigen to CTL

Kast et al. (37) reported that C57BL/6 (B6, H-2^b) mice are CTL responders to Sendai virus and Moloney leukemia virus. The H-2K^b class I MHC molecule is the CTL restriction molecule. In contrast, the H-2K^b mouse mutant *bml* does not generate CTL response to Sendai virus. The mouse H-2D^b mutant 14 does not generate the CTL response to Moloney virus but responds normally to Sendai virus. This specific CTL response defect was restored by syngeneic Moloney virus-infected dendritic cells (DC). The authors concluded that DC very effectively present

viral antigen to CTLs by a particular MHC class I-virus peptide combination.

Antigen-Presenting Dendritic Cells (DC) Promote CTL Growth

Macatonia et al. (38) showed that DC promote CTL growth from unprimed lymphocytes by using viral peptides or from primed lymphocytes by using whole viral antigen. Cherrie et al. (39) used DC from peripheral blood to present respiratory syncytial virus (RSV) antigen to autologous peripheral blood monocyctic cells that were obtained from normal donors. Reproducible CTL production in short-term cultures from all nine donors was found. These CTLs were tested against autologous Epstein-Barr virus-transformed lymphoblastoid cell lines, each infected with a different recombinant vaccinia virus, expressing one RSV gene only. It was found that CTLs recognized 6 of 10 virus-coded proteins and that the recognition was restricted by major histocompatibility complex (MHC) class I specificity.

Mouse Spleen Dendritic Cells Function to Generate Influenza-Specific CTLs

Nonacs et al. (40) reported that influenza virus-treated dendritic cells (DC) were 100 times more efficient than bulk spleen cells in stimulating CD8⁺ CTL formation without exogenous lymphokines. The infectious virus entered the DC through intracellular acidic vacuoles (endosomes) and replicated in these cells. Viral peptides derived from cytosol-processed viral proteins were presented by MHC class I molecules on the plasma membrane and generated CTLs. Dendritic cells pulsed with the MHC class I-restricted influenza virus nucleoprotein peptide NP(aa147–155) were poor stimulators in the absence of exogenous helper factors. It was concluded that viral protein synthesis in DC mediates the presentation of the viral peptides by the MHC class I molecules needed for the induction of influenza-specific CTLs. Langhoff and Steinman (41) showed that cloning of CD8⁺ or CD4⁺ T cells was possible in the presence of syngeneic or allogeneic DC.

DC Present Antigens to CD4⁺ T Helper Cells via MHC Class II Molecules

DC Presentation of Inactivated Influenza Virus Antigens

The studies by Nonacs et al. (40) and Langhoff and Steinman (41) showed that DC present antigens and stimulate both CD8⁺ CTLs and CD4⁺ T helper cells. It was noted (40) that ultraviolet-inactivated influenza virus or virus treated with the enzyme bromelain to degrade the virus hemagglutinin resulted in the uptake of the virus particles by endocytosis into endocytotic vacuoles. Acid-dependent degradation of the viral proteins leads to the generation of peptides in the endosomes that interact with the peptide binding groove on MHC class II molecules when the MHC class II carrier vacuole fuses with an endosome that contains the foreign protein. In the fused vesicle the II molecules are proteolytically cleaved and viral antigenic peptides in the MHC molecule are transported to the plasma membrane to be presented to CD4⁺ lymphocytes in association with MHC class II molecules.

Clustering of DC Helper T Lymphocytes and Histocompatible B Cells during Primary Antibody Responses In Vitro

Inaba et al. (42) isolated clusters of cells consisting of 20–50% B cells, 20–50% T cells, and 10–20% dendritic cells from mouse spleen suspensions. B cells cluster only in the presence of T cells and DC. These clusters are the site for development of plaque-forming cells. Sornasse et al. (43) showed that injection of DC, pulsed in vitro with a soluble antigen (myoglobin), induced specific humoral responses in vitro.

Lymph-Borne Dendritic Cells Acquire and Present Intestinally Administered Antigens

Liu and MacPherson (44) reported that injection of ovalbumin and horseradish peroxidase into the rat gut sensitized lymph-borne dendritic cells for presentation. The DC acquired the antigen within 8 h. The antigen-specific response was blocked by antibodies to CD4 and to MHC class II antigens.

Studies on the Molecular Basis of Presentation of Viral Peptides in Association with MHC Class I Molecules

Influenza Virus-Infected Cells Present a Virus Peptide on MHC Class I Molecule

Zinkernagel and Doherty (45) and Doherty and Zinkernagel (46) were first to demonstrate that viral antigens are presented in association with proteins coded by genes within MHC genes. Townsend et al. (47) demonstrated that cytotoxic T cells recognize fragments of the influenza virus nucleoprotein. To obtain information on the viral peptide that is presented to CTL by MHC class I molecules, Gotch et al. (48) prepared synthetic peptides modeled after the viral nucleoprotein and demonstrated that the synthetic influenza virus peptide NP335-349 was recognized in association with HLA-B by influenza-specific human CTL from a donor. The peptide was assayed by incubation with ⁵¹Cr-labeled autologous B lymphoblasts. A CTL line from an HLA-A2-positive donor infected with influenza virus was incubated with a matrix peptide amino acid (aa) 56–68 on lymphoblastoid cells. The CTL-line cells lysed only target cells that had been treated with the same aa56–68 peptide and that shared the HLA-A2 class I molecules. Elliot et al. (49) reported that peptides have two cooperative roles in the assembly of MHC class I heavy chain and the light chain β_2 -microglobulin (β_2m). Short (9aa) peptides can stabilize the association of the MHC class I heavy chain with β_2 and may be the basis for selection of peptide epitopes during in vitro presentation. When a dilute lysate of RMA-S cells was derived from Rauscher virus-transformed mouse lymphoma RMA of C57BL/6 A origin (50), it was found that these cells were defective in peptide presentation to the MHC class I heavy chain molecules. It was found that the heavy and light chains were predominantly dissociated, but that the addition of peptides led to a change in conformation of the heavy chains and to their association with β_2m molecules. The defect in peptide presentation by RMA-S cells was corrected by transfection of these cells with cDNA of one of the two genes coding for the peptide transporter proteins present in the membrane of the endoplasmic reticulum (51–56).

These channel proteins are involved in the transport of peptides from the cytoplasm to the lumen of the ER.

Effect of Temperature on the Presence of Empty MHC Class I Molecules Present on the Cell Membrane

Ljunggren et al. (57) studied the murine lymphoma mutant cell line RMA-S, which is mutated in one of the peptide pump genes and is incapable of assembling the heavy and light chains of the MHC class I molecules. These authors reported that despite the mutation, upon incubation of the RMA-S cells at 19–33°C, MHC class I molecules assemble in the ER lumen and are transported to the plasma membrane. They can be stabilized at 37°C by exposure of the cells to specific peptides known to interact with H-2K^b or D^b class I molecules.

Parham (58), in a review of the Ljunggren et al. (57) study, suggested that empty MHC class I molecules are present on many cell types and are possibly more efficient than "full" molecules, which carry self-peptides obtained during their assembly in the endoplasmic reticulum.

Peptide Presentation by DC to CD8⁺ T Cells

De Bruijn et al. (59) studied the ability of various antigen-presenting cell (APC) types to induce primary antiviral cytotoxic T lymphocyte (CTLs) responses by single in vitro stimulation. These authors report that only spleen dendritic cells (DC) from C57BL/6 mice RMA-S lymphoma cells were able to induce primary CTL responses by different mechanisms. The DC were able to present exogenous Sendai virus peptides and endogenous viral peptides (after virus infection) and induced primary virus-specific CTLs in vitro. In contrast, RMA-S cells were able to present exogenous viral peptides, but not endogenous viral peptides that were generated in the cytosol during infection of the cells, since the transport mechanism for the peptides is defective in these cells. It was also reported that the number of MHC class I molecules on the surface of DC are 30-fold more efficient than on RMA-S cells, while peptides binding to the latter cells is five times greater than to the DC.

The induction of the primary CTL response by DC and RMA-S cells was blocked by anti-LFA-1 and anti-CD8 monoclonal antibodies. DC rapidly aggregated with unprimed T cells. DC did not bind much exogenous viral peptides, while RMA-S cells were able to bind much more of the peptide. In a later stage of cell-to-cell contact, both DC and RMA-S cells activated LFA-1 and CD8 molecules at the T-cell surface to strengthen and maintain the contact between T cells and the antigen-presenting cell (59). For the RMA-S cells to be able to accept the exogenous peptide, the cells must be preincubated at 26°C. Under these conditions the MHC class I molecules are empty, but the heavy chains and the β_2 -microglobulins interact to form empty MHC class molecules, with empty peptide binding grooves, which are transported to the surface of the RMA-S plasma membrane, as reported by Ljunggren et al. (57). This may explain the five-fold difference between the ability of DC and RMA-S cells to bind exogenous peptide reported by De Bruijn et al. (59). The authors suggested that only some of the MHC class I molecules on the surface of DC are empty or that the exogenous peptide needs to remove an endogenous self-peptide bound to the peptide binding groove of MHC class I molecules before it can bind.

Properties of the Peptide Binding Groove (Pocket) in MHC Class I Molecules Determined by x-ray Crystallography

The studies by Bjorkman et al. (60) and Bjorkman and Davis (61) enhanced the understanding of the structure and organization of the peptide binding groove in the heavy chain of the MHC class I molecule. Eventually these studies led to the characterization of self and nonself peptides that are associated with the MHC class I molecule peptide binding groove.

Bjorkman et al. (62) defined two domains with immunoglobulin folds in the membrane proximal end of the HLA class I glycoprotein that are paired in a novel manner. The region in the molecule distal from the membrane is a platform of eight antiparallel β -strands topped by two α -helices. A large groove between the α -helices provides a binding site for the antigenic peptide. The groove is about 25 Å long, 10 Å wide, and 12 Å deep. The sides of the groove are

formed by the side chains from the amino acids in the α -helices and the β -sheets in the floor of the groove.

A refined analysis of HLA-A2 Saper et al. (63) provided a detailed analysis of the peptide binding groove and demonstrated that the solvent-accessible amino acid side chains that are most polymorphic in mouse and human alleles fill up the central and widest portion of the peptide binding groove. The conserved side chains are clustered at the narrower ends of the peptide groove. The amino acid side chains in the peptide groove form six pockets (subsites) of diverse shapes and composition, suited for binding of side chains of the amino acids in the antigenic peptide. Three of the pockets contain predominantly nonpolar atoms, but the others, especially the two pockets at the extreme ends of the groove, have clusters of polar atoms.

A detailed analysis (63) of each of the six pockets for the nonameric antigen peptide suggested the mode of interaction of the peptide amino acid side chains with the pockets in the peptide binding groove of HLA-A2. This analysis showed that mutations in the nucleotide sequence of the MHC class I heavy chain gene will lead to changes in the amino acids that form the "walls" of the peptide binding groove. Hence, such mutations will lead to modifications in the antigenic peptides that interact with the polymorphic peptide binding grooves in individuals with different haplotypes. Van Bleck and Nathenson (64) examined the effect of diversity in antigen binding grooves and noted that mutations in the HLA types determine the selection of self-peptides.

Presentation of the Antigenic Peptide in the Peptide Groove MHC Class I Molecules to the T-Cell Receptors (TCRs) on CD8⁺ CTLs

The sequence data suggested (61) that the TCR V regions located on loops are folded into a β -sandwich structure resembling immunoglobulin V regions. TCR V domains will not only fold into tertiary structures similar to the antibody V region but the chain pairing and resulting combining sites of TCR will also be similar to those described for antibodies. Bjorkman and Davis (61) suggested a model for TCR recognition of

the peptide-MHC complex by comparing the hypothetical combining site of the TCR to the top surface of the peptide in the peptide binding groove of the MHC class I molecule. These authors noted that the α -helices that make up the sides of the peptide groove are separated by about the same distance (18 Å) that separates the CDR1 and CDR2 regions of one V domain from the other. Thus, the relatively flat surface of the peptide/MHC class I complex may interact with the combining site of an immunoglobulinlike TCR, as the limited diversity in CDR1- and CDR2-equivalent regions on TCR V α and V β contacts the side chains of the MHC α -helices, leaving the centrally located and very diverse CDR3-equivalent regions to interact with the antigenic peptide.

MHC Class I Molecule Presenting Viral Peptides

Silver et al. (65) described the structure of the complex between class I histocompatibility glycoprotein HLA-Aw68 and an influenza virus nucleoprotein-derived peptide NP91-99 determined by x-ray crystallography. The authors reported that the amino acid residues at both ends of the peptide are buried inside the peptide binding groove, while the amino acids in the middle of the peptide (aa4-8) are exposed and could be recognized by the T-cell receptor. Zhang et al. (66) studied the crystal structure of MHC class I H-2 K^b molecules containing an octapeptide designed according to vesicular stomatitis virus (VSV) nuclear capsid protein aa52-59. It was found that all residues of the octapeptide were in a β -strand conformation and that the N-terminal amino group of the peptide was oriented toward the interior of the groove and formed a hydrogen bond with the hydroxyl group of tyrosine 171 of H-2K. The number of residues in the peptides that bind H-2Kb MHC class I molecules was eight or nine, while the VSV peptide contained eight residues.

Characterization of Self and Viral Peptides Bound to HLA Class I Haplotypes

Jardetzky et al. (67) identified the peptides bound to HLA-B27 molecules as a nonamer with a simi-

lar motif: arginine in position 2 and an arginine or lysine in position 9. A similar motif was found in a peptide derived from influenza virus A nucleoprotein (aa383–391), HIV-1 Gag p24 (aa265–276), and HIV-1 gp120 (aa314–322). Madden et al. (68) identified self-peptides bound to the peptide binding groove of class I HLA-B27 in an extended conformation.

Hunt et al. (69,70) reported that in HLA-A 2.1 the peptide binding groove is hydrophobic and is capable of accommodating leucine or isoleucine side chains. Guo et al. (71) reported a refined structure at a resolution of 1.9 Å by x-ray crystallography of HLA-Aw68, showing the atomic structure of the first three and last two amino acids in the bound peptide, but no connected electron density in between could be resolved. The authors suggested that the unresolved central amino acids of the antigenic peptide bulge from the peptide binding groove, which retains the terminal amino acids of the antigenic peptide. Eluted peptides include peptides with 9, 10, and 11 amino acids with a distinct motif: The second amino acid was valine and the last arginine or lysine. Influenza virus peptide NP 91–99 conforms with the motif of HLA-Aw68.

Wei and Cresswell (72) reported that the mutant human cell line T2 is defective in antigen presentation and shows poor surface expression of exogenous HLA alleles. HLA-A2 molecules on the T2 cell membrane bound high levels of a limited set of endogenous peptides, of which three peptides were sequenced. Two of the peptides containing 9aa and one that contained 11aa were derived from the putative signal sequence of the cellular IP-30 protein, an interferon- γ -inducible protein. The third peptide contained 13aa.

Peptides in the Peptide Binding Groove of Mouse MHC Class I Molecules

Schumacher et al. (73) studied synthetic peptides captured by "empty" class I molecules of mutant cell line RMA-S and reported that 9-mer peptides selectively bind to MHC class I molecules, even when they are a minor component in a mixture of longer peptides. Falk et al. (74) determined the sequence of peptides derived

from HLA-A2 and mouse K^d, D^d, and K^b molecules and determined the dominant anchor, and the strong and weak amino acids with respect to the binding to the peptide binding groove.

Peptide Binding Site of Class II Histocompatibility Molecules and the Properties of Antigenic Peptides Capable of Binding

Organization of the Peptide Binding Groove of MHC Class II Molecules

On the basis of the nucleotide sequence of the gene or heavy chain of HLA-DR antigen and its homology to immunoglobulins (75), Brown et al. (76) suggested a hypothetical model of the peptide binding groove in class II MHC molecules. The two polypeptide chains of class II molecules α and β are each composed of two domains. The N-terminal domains of each $\alpha_1\beta_1$ are highly polymorphic and appear responsible for binding peptides at a single site that is recognized by MHC-restricted antigen-specific T cells. The model provides a structural framework for proposing a peptide binding model. Berzofsky et al. (77) suggested a model of the peptide binding domain on MHC class II molecules in which the α_1 and β_1 α -helical regions constitute the walls of the peptide binding domain and β -sheets of the β_1 region make the bottom of the domain. From this model it can be noted that the peptide binding domain is open ended. Rothbard et al. (78) reported changes in the amino acid sequence of the β_1 domains of HLA-DR1, DR4DW, and D7DW7 class II molecules. Recently, Brown et al. (79) reported the three-dimensional organization of the HLA-DR1 peptide binding groove and the mode of its interaction with antigenic peptides.

Properties of the Peptides that Bind to the Peptide Binding Groove on Class II MHC Molecules

Budensky et al. (80) determined the sequence of peptides bound to MHC class II molecules and suggested that the binding domain of MHC class II molecules is open on one side and may accommodate longer peptides, which are possibly trimmed by carboxypeptidase to conform with

the correct size of the antigenic peptides. Budensky et al. (81) sequenced self-peptides extracted from MHC class II molecules and determined the motifs in the peptides. The sequence motif indicates that certain amino acid residues occur at particular positions in the sequence.

Peptides Derived from Exogenous and Endogenous Proteins Become Associated with MHC Class II Molecules in the Endosome Pathway

Hunt et al. (69) reported that all the peptides present in association with class II molecules were 16–18 residues long and contained a six-residue binding motif that was variably placed within the peptide chain. Binding data on truncated peptides suggested that the peptide binding groove of class II molecules can be open at both ends. The peptides derived mainly from exogenous proteins that enter the cell by phagocytosis, endocytosis, or internalization of the cell membrane are processed in acidic endosomal compartments.

Riderby et al. (82) reported that HLA-DR3 molecules isolated from T2 transfectant cells were efficiently loaded with antigenic peptides by exposure to a low pH in vitro. However, in vivo such MHC class II molecules are in association with a nested set of peptides derived by proteolysis of the invariant chain (Ii). The invariant chain associates with the MHC class II molecule heterodimer in the membrane of the endoplasmic reticulum and prevents the class II molecules from interaction with nonameric peptides during the period when these molecules reside in the endoplasmic reticulum lumen. The Ii molecules contain an amino acid signal sequence that targets the MHC class II molecule-invariant chain complex to an acidic endosomal compartment in the cytoplasm. A vesicle is formed that is transported to the endosomal compartment.

Proteolytic cleavage and dissociation of the invariant chain takes place when the vesicle that carries the MHC class II-Ii complexes fuses with an endosome in the endosome pathway, allowing the cathepsin in the endosome to proteolytically cleave the Ii polypeptide, releasing the MHC class II molecules from Ii. The class II molecules fold and interact at a lower pH with peptides in the endosome that are the proteolytic products

of foreign or self-proteins. The time course from synthesis of the class II molecules to transport in the cells to the cell surface was reported by Cresswell et al. (83).

Type C Nerve Fiber Neurotransmitter Calcitonin Gene-Regulated Peptide Affects Antigen Presentation by Professional Antigen-Presenting Cells: Skin Langerhans Cells and Macrophages

Properties of the Neurotransmitter CGRP and Its Antagonists

Calcitonin gene-related peptide (CGRP) is a 37 amino acid long peptide produced by alternative processing of the RNA transcript from the calcitonin gene (84). Rosenfeld et al. (85) reported that "the distributions of CGRP-producing cells and pathway in the brain, spinal cord, ganglia and other tissues like the medulla of the adrenal gland suggests functions for the neuropeptide in nociception, ingestive behavior and modulation of the autonomic and endocrine systems." An important role of CGRP is in the processing of painful stimuli (85).

CGRP (which in humans exists as two forms, α and β , differing in three amino acids) exerts its effect by interaction with specific receptor types 1 and 2. Studies on the structure of CGRP revealed the presence of a disulfide bonded loop at the N-terminus adjacent to the alpha helical segment in amino acids 8–18. Modeling suggested a turn immediately after the alpha-helix in amino acid residues 19–23 and 29–43. For type 1 receptors an intact disulfide bond is required for biological activity, but a linear CGRP analog lacking the disulfide is active on type 2 receptor. The major determinants for binding to receptors reside in amino acids 8–37, which are an antagonist. It was reported that a CGRP lacking its C-terminal phenylalanine residue was unable to bind to its receptor (86).

CGRP Interacts with Skin Dendritic (Langerhans) Cells and Inhibits Antigen Presentation by LC and Macrophages

CGRP in skin nerve endings. The skin receives a rich supply of nerve fibers, and substance P

immunoreactivity was demonstrated in nerve fibers along blood vessels and as free nerve endings in the dermis and epidermis. CGRP was demonstrated in primary sensory neurons containing substance P (87). In the latter study, nerve fibers storing substance P and CGRP were present as free nerve endings in the dermis and epidermis of the human skin. Delsgaard et al. (88) reported that the majority of the CGRP immunoreactive sensory fibers present in the skin epidermis and dermis, around blood vessels and hair follicles, also displayed substance P immunoreactivity. No nerve fibers displaying substance P and somatostatin were found, but a few substance P nerve fibers in the dermis-epidermis region were found to also contain vasointestinal polypeptide immunoreactivity. Alvarez et al. (89) reported that CGRP and substance P are present in nerve endings penetrating the epidermis of the cat nose and also in the snouts of hedgehogs.

CGRP in esophageal and skin Langerhans cells. Singaram et al. (90) reported that CGRP-immunoreactive intraepithelial cells previously seen in human esophageal mucosa were identified as intraepithelial Langerhans cells with specific antibodies to Langerhans cell markers (Ia, HLA-DR, and OKT6). The authors suggested that CGRP may serve as an immunomodulator or a cytokine, since CGRP affects the mitogenic response of human monocytes stimulated with phytohemagglutinin. (Casini et al. quoted by Singaram et al. (90)). Hosoi et al. (91) reported that CGRP-containing nerve fibers are intimately associated with Langerhans cells in human epidermis, and CGRP was found at the surface of some Langerhans cells.

CGRP Inhibits Antigen Presentation by Langerhans Cells and Macrophages

Hosoi et al. (91) investigated the effect of CGRP on antigen presentation by skin Langerhans cells and demonstrated by three functional assays that CGRP inhibited presentation by these cells. This finding was taken to suggest that CGRP plays an important role in the modulation of antigen presentation by macrophages.

CGRP Inhibits H_2O_2 Production by Macrophages

Nong et al. (92) indicated that CGRP was found to inhibit the ability of macrophages to produce H_2O_2 in response to interferon γ or to act as antigen-presenting cells. This finding was taken to suggest that CGRP plays an important role in modulation of antigen presentation by macrophages.

Will It Be Possible to Induce Antiviral CTLs by Synthetic Viral Peptides Introduced to Skin LC In Vivo?

Review of the current knowledge on Langerhans cells clearly shows that they are excellent antigen-presenting cells that efficiently present viral peptides to $CD8^+$ T cells, thus priming the development of cytotoxic T cells and antigenic viral peptides to $CD4^+$ T cells, leading to the synthesis of antiviral antibodies. Indeed, LC were found to form clusters of T helper and B lymphocytes in lymph nodes. The LC express on the cell plasma membrane CD1, MHC class I, and MHC class II molecules, which are able to present endogenously (after processing in the cytosol or endosomes, respectively) and exogenously processed viral proteins, as well as peptides, derived from viral proteins during virus (e.g., influenza virus matrix protein) replication in the antigen-presenting cell.

It was also found that "empty" MHC class I and class II molecules are present on the cell membranes of DC, which may be capable of taking up synthetic peptides into the peptide binding grooves. Indeed, subcutaneous injection into mice of a lymphocytic choriomeningitis (LCM) virus synthetic peptide was reported to induce antiviral protection by cytotoxic T cells (93). Studies in our laboratory showed that subcutaneous injection of a viral synthetic peptide resulted in the binding of the peptide to Langerhans cells and the migration of peptide-carrying DC to regional lymph nodes (Becker, Yadin, and David, to be published).

The ability of MHC or HLA molecules on the plasma membrane of LC to interact with viral peptides may result from their position in skin at

a temperature lower than 37°C, which might allow the assembly of MHC class I molecules without the need for insertion of peptides into the peptide binding groove. Such empty MHC or HLA class I molecules may be available for interaction with synthetic viral peptides designed according to the peptide motif of the HLA haplotype to prime cytotoxic T cells. Insertion of HLA viral peptides designed according to the haplotype known to induce CD8⁺ CTLs in infected individuals may lead to a new approach to cellular immunization to protect humans against virus infections in which antiviral antibodies aggravate the viral disease. These peptide-primed CTLs recognize and destroy virus-infected cells that present the viral peptides on HLA class I molecules in an infected individual. Such a CTL response will prevent virus replication as well as enhancement of the virus infection due to antiviral antibodies and virion interaction due to virus attachment to Fc receptors on the host cells (39).

Alternatively, it should be taken into consideration that longer synthetic peptides, which include the nonameric peptides, might be introduced into the skin. Such peptides may be processed by the LC proteolytic enzymes to release the correct nonameric peptides that will fit the HLA class I peptide binding groove.

It was reported by Takahashi et al. from Berzofsky's group (94) that "a single subcutaneous immunization in mice with immunostimulating complexes, containing either purified, intact gp160 envelope glycoprotein of the human immunodeficiency virus (HIV)-1 or influenza hemagglutinin, results in reproducible and long lasting priming of HIV-specific or influenza-specific CD8⁺ class I restricted CTL" (94). The injection of the viral protein immunostimulating complexes into the subcutaneous compartment of the skin seems to bring the viral proteins to the skin Langerhans cells, which present the processed viral proteins by MHC class I molecules to CD8⁺ T cells, thus priming the antiviral cellular immune response.

Introduction of Bioreactive Peptides into the Skin

Banerjee and Ritschel reported (95) on the influence of pH, peptide concentration, sharing, and

surfactant on the in vitro transdermal permeation of vasopressin. They reported that a higher transdermal permeation of vasopressin was noted at pH 5.0 at a concentration of 49.5 µg/ml. It was also noted that treating with 25 cellophane tape strippings caused a 70 times increase in transdermal permeation as compared with untreated skin. Cellophane tape stripping of the skin is known to inactivate skin LC, indicating that the skin LC are a barrier to transdermal transport of peptide through the skin, which is the purpose of drug delivery through the skin into the skin dermal vascular network. For transepidermal delivery of immunoreactive peptides to the skin LC, the method reported by Banerjee and Ritschel (95) may suffice.

Choi et al. (96) investigated the effects of the nonionic surfactant, n-decylmethyl sulfoxide (NDMS), and pH on the permeation of the pentapeptide enkephalin through hairless mouse skin. It was reported that after permeation of the enkephalin the peptide (YGGFL) was rapidly cleaved by a peptidase at the Tyr-Gly bond. At pH 5.0 the metabolic activity was significantly reduced and a substantial flux of YGGFL was observed. The authors concluded that a combination of a skin permeation enhancer, pH adjustment, and inhibitors of proteolytic activity in the skin can increase transdermal delivery of peptides.

Transepidermal Transport of HLA Class I-Related Synthetic Viral Immunogenic Peptides Targeted to the Skin Langerhans Cells

Based on available knowledge on the transport of bioreactive peptide through the skin and the possible role of LC in inhibiting their transport from the epidermis to the dermis, it is possible to suggest that under appropriate conditions the viral synthetic peptides will be introduced to the Langerhans cells in the skin. LC, being professional antigen-presenting cells, will use the peptides for presentation to CD8⁺ T cells in the lymph nodes. The following aspects of the "Peplotion" concept should be considered:

1. Optimal conditions for transepidermal transport of the viral peptides in the human skin, since the available data are on mouse skin
2. HLA class I types and haplotypes of the

human population must be determined prior to immunization, and the motifs of the nonapeptide that bind to their HLA-class I peptide binding groove are known

3. Design of the viral nonapeptides to fit the peptide binding grooves of HLA class I molecules of the human population
4. Influence of the nervous system on the immune response in the skin

For each virus the sequence of the nonapeptide that induces CTLs must be known. This information may be obtained by extracting the peptides from the surface HLA class I molecules of infected cells, sequencing each peptide, and comparing the amino acid sequence to the sequence of the viral proteins. Alternatively, since the motifs of the viral nonapeptide are selected by the HLA type of the individual, computer modeling of the peptide binding grooves of different HLA subtypes (97), as well as modeling of the nonapeptides (98), may provide the necessary information for the design of viral peptides. This information is critical for designing viral nonapeptides that will interact with the specific peptide binding groove of HLA types. Different populations display different HLA haplotypes, which must be considered when a synthetic peptide vaccine is considered for priming antiviral CTLs.

The "Peplotion" approach may also use the ability of LC HLA class II molecules to interact with longer antigenic viral peptides that are presented to CD4⁺ T helper cells and induce specific neutralizing antibodies. Such antiviral antibodies may be useful in immunizing against virus infections against which antiviral antibodies protect. The concept of using skin LC for the priming of T cells may be expanded to the use of gut dendritic cells and lung dendritic cells to prime CTLs or CD4⁺ T cells in the gut or lung, respectively. Such an approach may provide enhancement of the immune responses in different organs of the body and may lead to more successful elimination of virus infections in humans and economically important animals.

Recent studies revealed the ability of the central nervous system to regulate the release of CGRP into the skin and epithelium of mucous membranes. The presence of receptors to CGRP

on professional antigen-presenting cells (Langerhans cells and macrophages) indicates that modulation of the release of CGRP from nerve endings in the skin may affect antigen presentation by these cells (92). The connection between CGRP release in the skin and the immune response to a virus vaccine introduced by injection into the skin is not yet known. Further studies are needed to determine if immunization by a viral peptide ("Peplotion") vaccine will require the use of CGRP antagonist to enhance the ability of Langerhans cells to efficiently present the viral antigens to T cells.

Acknowledgments

The author's research on dendritic cells is supported by a grant from the Israel-USA Binational Foundation. Continued support from the Foundation for the Study of Molecular Virology and Cell Biology, Phoenix, Arizona, is greatly appreciated. The author wishes to thank Prof. Charles DeLisi, Dean, College of Engineering, Boston University, for his hospitality during the author's sabbatical as a Distinguished Visiting Scientist at the College of Engineering, Boston University, Boston (May to August, 1993) and Dr. Rakefet Rosenfeld, Department of Bioengineering, College of Engineering, Boston University, for her cooperation. The help of Mrs. Yael Asher and Eynat Tabor in computer analyses of HIV proteins, and of Mrs. Cheryl Berkowitz-Balshayi and Mrs. Frances Birnbaum in the preparation of the manuscript, is gratefully acknowledged.

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